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Advertisement calls of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis* (Anura, Megophryidae)

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Abstract

In this study, the advertisement calls of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis* are described. The advertisement call of *L. suiyangensis* includes simple and complex calls, with four different call types and a dominant frequency ranging 4.13–4.82 kHz. The advertisement call of *L. bashaensis* consists of a single note, with a dominant frequency 6.03–6.46 kHz. We compare the advertisement calls with other species in the genus *Leptobrachella*, and discuss the definitions of primary advertisement calls and secondary advertisement calls. Our results provide basic data for further acoustic, taxonomic and ecological studies in the genus *Leptobrachella*.

Key Words

acoustic differences, bioacoustics, frogs, southern China

Introduction

In anurans, acoustic communication is the most important form of communication at the interspecific and intraspecific levels, playing an important role in species reproduction, evolution and interspecific identification (Cunningham and Birkhead 1998; Brenowitz and Rose 1999; Kelley 2004). For further understanding the relationship between the behaviors and vocal communication in frogs, researchers have divided frog calls into the following four types: reproductive calls, aggressive calls, defensive calls, and feeding calls. Reproductive calls include advertisement calls, courtship calls, amplectant calls, release calls, post-oviposition male release calls, and rain calls (Toledo et al. 2015; Köhler et al. 2017). Advertisement calls are not only the main vocal type of frogs, but also vary greatly among different species, so they can be used as a basis for systematic classification and identification of cryptic species (Sullivan et al. 1996; Rowley et al. 2015).

The Asian leaf litter toads of the genus *Leptobrachella* (Smith, 1925) are a group of forest-dependent species, widely distributed in Southeast Asia, southern China, and northeast India (AmphibiaChina 2023; Frost 2023). *Leptobrachella* often inhabits the rocks on the stream banks during the breeding season. Several *Leptobrachella* species are threatened with extinction, 22.2% of them are listed as critically endangered (CR) or endangered (EN) in the IUCN Red Species List, such as the critically endangered *L. botsfordi* and *L. kecil* (IUCN 2021). High levels of morphological similarity and rampant homoplasy appear to have misled estimates of diversity and evolutionary relationships (Chen et al. 2018). Thirty-seven species of *Leptobrachella* have been described in the last five years, representing 37.8% of the total number in this genus (AmphibiaChina 2023; Frost 2023). Despite

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this considerable number of discoveries and publications, the vocalizations of many *Leptobrachella* species remain unknown (Yeung et al. 2021). Both *L. suiyangensis* and *L. bashaensis* were described in 2020 (Luo et al. 2020; Lyu et al. 2020), but their advertisement calls have not been reported so far. In this study, we describe for the first time advertisement calls of *L. suiyangensis* and *L. bashaensis*. Furthermore, we also compare these calls with other species in the genus *Leptobrachella* from literature (Suppl. material 1), in order to provide basic data for further acoustic, taxonomic and ecological studies in the genus *Leptobrachella*.

Materials and methods

Call recordings

Our experimental procedures complied with the applicable laws on animal welfare and research in China and were approved by the Subcommittee on Experimental Animal Ethics of Guizhou University (Permit No. EAE-GZU 2023-E013).

The advertisement calls of *L. suiyangensis* and *L. bashaensis* were both recorded from their type localities Suiyang County and Congjiang County, Guizhou Province, China. A total of 322 calls were recorded from four individuals of *L. suiyangensis*, collected from Huoqiuba Nature Reserve (107.08°E, 28.47°N, ca. 1450 m elev., 15.7 °C air temperature, 93% ambient humidity) on April 27, 2022, between 19:00–23:00 h, and 100 calls from three *L. bashaensis* individuals collected from Basha Nature Reserve (25.63°N, 108.39°E, ca. 980 m elev., 16.3 °C air temperature, 83% ambient humidity) on May 1, 2022, between 19:00–20:00 h. Calls of each individual were obtained using a digital recorder, SONY ICD-PX470 (sampling rate 44.1 kHz, 16-bit resolution). Each call was recorded within a 0.5 m distance from the calling individual. Recorded calls were always of isolated individuals and never from a mixed chorus. The recordings were saved as WAV files. The recordings are publicly available in Figshare at https://doi.org/10.6084/m9.figshare.24147255. Snout vent lengths (SVLs) of all recorded males were measured in situ using a precision digital calliper to the nearest 0.1 mm. One *L. suiyangensis* (specimen number: SY20220427003) and one *L. bashaensis* (specimen number: CJ20220501001) were collected for species identification and the others were released to their original habitat after measurement. After taking photographs, they were euthanized using isoflurane and then the specimens were fixed in 10% buffered formalin. Tissue samples were taken and preserved separately in 95% ethanol before fixation. Specimens were deposited in the Forestry College of Guizhou University, China. Mitochondrial 16S rRNA genes were extracted and amplified from muscle samples of all samples and sequenced, and the obtained sequence was verified and uploaded to Genbank (https://www.ncbi.nlm.nih.gov/). The sequencing results were compared to finally determine the species collected. For the morphological identification, the procedure described by Luo et al. (2020) and Lyu et al. (2020) was followed.

Acoustic analyses

The advertisement call characteristics were analyzed with the software Raven Pro 1.6. (K. Lisa Yang Center for Conservation Bioacoustics at the Cornell Lab of Ornithology 2023). Temporal properties were measured using Raven's waveform display. Spectral properties were measured by averaging the spectrum over the entire duration of a call (Hann window, DFT = 512 samples, overlap = 50%, Hop Size = 256 samples). Only calls that had a high signal-to-noise ratio and were free from overlapping calls of nearby males were used for the analysis. We used "note-centered" terminology as summarized by Köhler et al. (2017), in which the fundamental unit was defined as a "note" and each "call" contains a single "note" or "note series". We measured all parameters and characteristics following the procedure described by Köhler et al. (2017) and Yeung et al. (2021) including (1) call duration (ms), CD (2) inter-call intervals, CI (3) call rate (calls/minute), CR (4) note per call, NPC (5) first note duration, first ND (6) second note duration, second ND (7) inter-note intervals, NI (8) note rate (notes/s) (9) first note pulse number, first NP (10) second note pulse number, second NP (11) pulse rate (pulses/s), PR (12) dominant frequency (kHz), DF (Table 1). Oscillograms, spectrograms, and power spectra were generated using Seewave v.2.2.0 (Sueur et al. 2008) and TuneR 1.4.2 (Ligges et al. 2013) packages in R programme 4.2.2 (R Core Team 2021) with a "Hanning" window size of 256 samples and an overlap of 50%. Descriptive statistics of call characteristics: mean, standard deviation (SD), and range were computed using SPSS 23.0. Furthermore, principal component analysis (PCA) was conducted to highlight whether the different types of advertisement calls of *L. suiyangensis* were separated in space.

Table 1. Descriptions of acoustic parameters measured.

Parameter (units)	Description
Call duration (ms)	The time between onset of first pulse and offset of the last pulse in a call.
Inter-call intervals (ms)	The time interval between two adjacent calls.
Call (repetition) rate	Instantaneous call rate. Number of calls emitted in a defined period.
Notes per call	The number of notes contained in a call.
First note duration (ms)	The duration of the first note in a complex call.
Second note duration (ms)	The duration of the second note in a complex call.
Inter-note intervals (ms)	The time interval between two adjacent notes.
Note (repetition) rate	Number of notes repeated within a defined period within a call or note series.
First note pulse number	The number of pulses contained in the first note of a complex call.
Second note pulse number	The number of pulses contained in the second note of a complex call.
Pulse (repetition) rate	Instantaneous pulse rate. Number of pulses repeated in a defined period within a note.
Dominant frequency (kHz)	Maximum frequency using Raven's selection spectrum function throughout the entire call.

Results

Leptobrachella suiyangensis Luo, Xiao, Gao & Zhou, 2020 (Fig. 1A)

The recorded males were calling on rocks in streams, with shrubs and bamboo forests growing nearby. After molecular identification, we determined that the species captured in the Huoqiuba Nature Reserve was *L. suiyangensis*. The 16S rRNA of the GenBank accession number is OR398777 (specimen number: SY20220427003). The SVL of *L. suiyangensis* ranged from 27.6 mm to 31.6 mm (Table 2). Based on parameters such as call duration, call structure and pulse structure of the advertisement calls of *L. suiyangensis*, we divide its advertisement calls into four types (Fig. 2; Table 3). In the PCA for the *L. suiyangensis* advertisement call type, the total variation of the first two principal components was 82.55%. The PC1 axis explained 59.83% of the total variation, mostly by the number of pulses per call (factor value = 0.987) and the pulse rate (factor value = 0.939); the PC2 axis was explained by the dominant frequency (factor value = 0.946), which explained 22.72% of the variance (Table 4). On the plot of PC1 vs. PC2, the four types of advertisement calls can be distinguished (Fig. 3). Although there is some overlap between call type C and type D, they can be defined by the difference in pulse numbers per note. Both type A (n = 3) and type B (n = 136) advertisement calls contained a single note, while type C (n = 138) and type D (n = 45) advertisement calls contained note series, and each note series contained two notes (Fig. 2). Among the four types of advertisement calls, the type D calls had the longest call duration, while the type A calls had the shortest call duration (Table 3). Type A, type B and type C advertisement calls had the same dominant frequency range in a call series. The dominant frequency of type D was slightly higher than the other three call types (Table 3).

Leptobrachella bashaensis Lyu, Dai, Wei, He, Yuan, Shi, Zhou, Ran, Kuang, Guo, Wei & Yuan, 2020 (Fig. 1B)

Recorded males perched on shrubs 0.5–1 m above the ground or were calling on rocks in streams. After molecular identification, we determined that the species captured in the Basha Nature Reserve is the *L. bashaensis*. The 16S rRNA of GenBank accession number is OR398776 (specimen number: CJ20220501001). The SVL of *L. bashaensis* ranged from 25.2 mm to 26.2 mm (Table 2). The advertisement call of *L. bashaensis* comprised a single note with a mean call duration of 66.01 ± 6.86 ms (Fig. 4; Table 5). The mean inter-call interval was 334.59 ± 65.61 ms. The call rate was 153.43 ± 9.16 calls/minute. The mean pulse number was 3.00 ± 1.00, with a mean pulse rate of 34.40 ± 4.46 pulses/second. The mean dominant frequency was 6.16 ± 0.08 kHz, and no obvious harmonics were found.

Table 2. Measurement of SVL of samples of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis*.

Species	Vouchers	SVL (mm)
Leptobrachella suiyangensis	SY20220427000	29.0
L. suiyangensis	SY20220427003	31.6
L. suiyangensis	SY20220427004	27.6
L. suiyangensis	SY20220427005	27.7
L. bashaensis	CJ20220501001	25.2
L. bashaensis	CJ20220501003	26.2
L. bashaensis	CJ20220501005	25.3

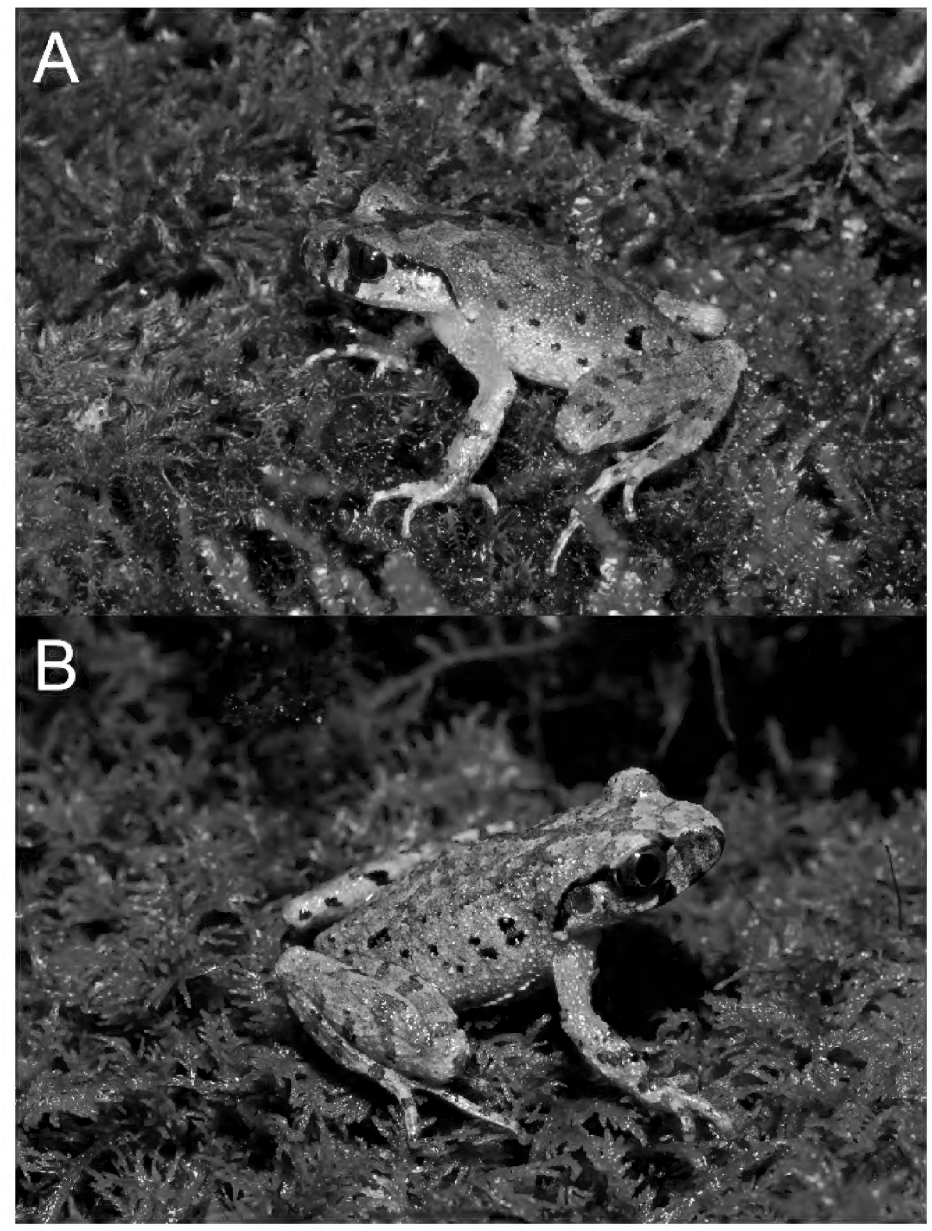

Figure 1. Photograph of the specimen. **A.** Adult male of *Leptobrachella suiyangensis* (SY20220427003, SVL 31.6 mm) from Huoqiuba Nature Reserve, Guizhou Province, China; **B.** Adult male of *Leptobrachella bashaensis* (CJ20220501001, SVL 25.2 mm) from Basha Nature Reserve, Guizhou Province, China.

Discussion

We describe for the first time the spectral and temporal parameters of the advertisement call of *L. suiyangensis* and *L. bashaensis*. The advertisement calls of *L. bashaensis* were simpler, with fewer pulse numbers. In addition, the dominant frequency in *L. suiyangensis* was significantly lower than that of *L. bashaensis*.

Among cryptic species, the use of acoustic diagnostic features for identification could be a potential alternative to morphometric and molecular diagnosis (Köhler et al. 2005; Vences and Köhler 2008). Despite their morphological similarities, *L. suiyangensis* and *L. bashaensis* exhibit different acoustic features in their calls, supporting the

species-specificity of their acoustic signals. However, the extent of cryptic diversity and the characteristics of their advertisement calls remain largely unexplored.

Related studies have shown that the evolution of advertisement call traits may be explained by historical (phylogeny), intrinsic (body size, exclusively male parental care), and extrinsic (calling site) factors (Bosch and De la Riva 2004; Mclean et al. 2013; Escalona et al. 2018). From the perspective of phylogenetic relationships, *L. suiyangensis* was assigned to the *L. oshanensis* species group (Luo et al. 2020; Liu et al. 2023). *Leptobrachella bashaensis* is nested in the *L. liui* species group. Previous studies have shown that the species in the *L. oshanensis* species group have more complex advertisement calls (Rowley et al. 2013; Wang et al. 2019; Shi et al. 2021; Suppl. material 1). In contrast, the advertisement call types of *L. liui* and *L. bashaensis* are simpler (Ding et al. 2019; Suppl. material 1). The genetic distance between the *L. bashaensis* and *L. oshanensis* species group is large, and they are not in the same branch (Liu et al. 2023).

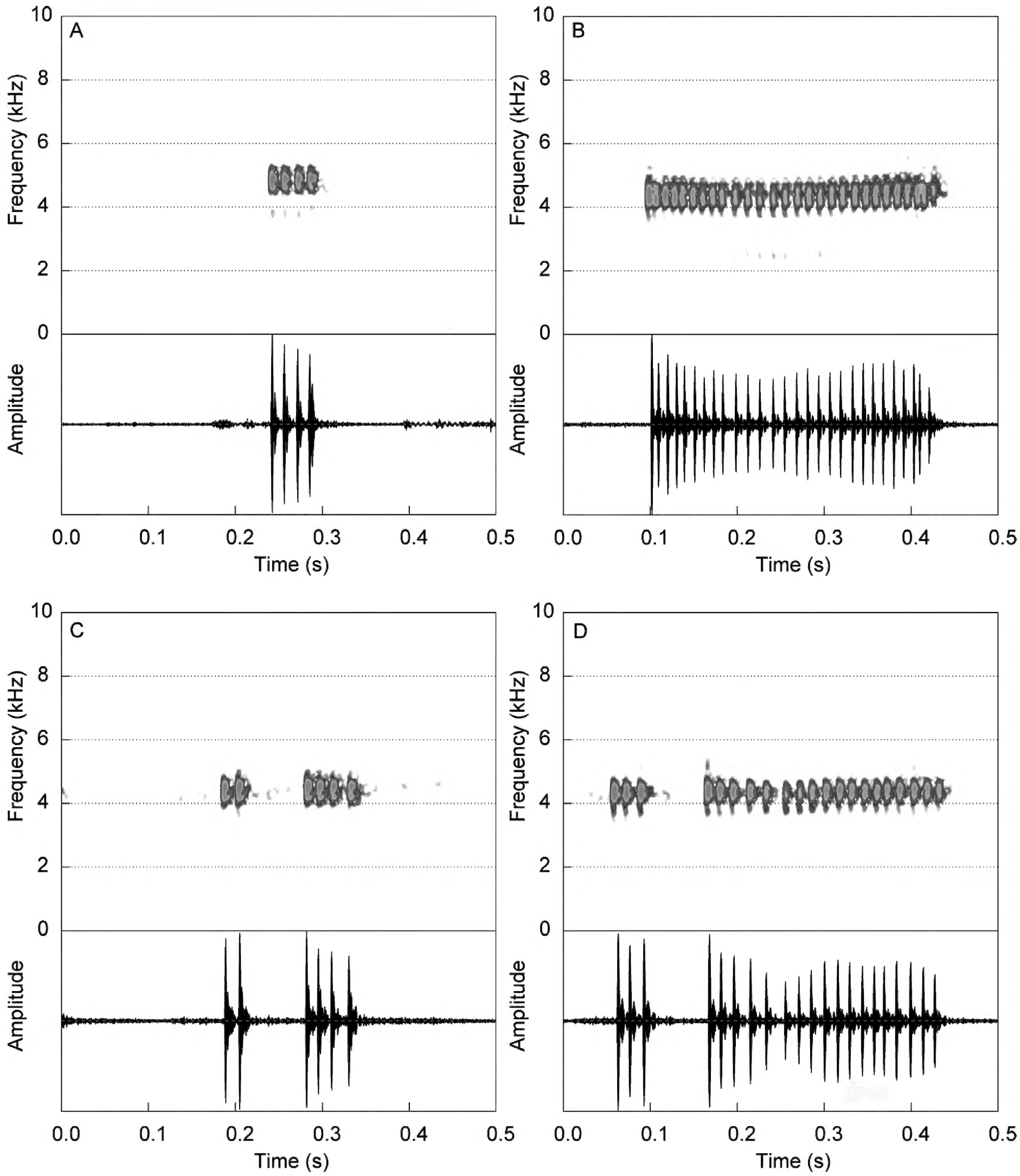

Figure 2. Oscillogram, spectrogram of four types of advertisement call of the *Leptobrachella suiyangensis* (SY20220427003, SVL 31.6 mm, 15.7 °C air temperature, 93% ambient humidity). **A.** 0.5 second type A advertisement call; **B.** 0.5 second type B advertisement call; **C.** 0.5 second type C advertisement call; **D.** 0.5 second type D advertisement call.

Table 3. Descriptive statistics for acoustic characteristics of advertisement calls of *Leptobrachella suiyangensis*. NA = not applicable.

11.75 mm	Vouchers	SY20220427000	SY20220427003	SY20220427004	SY20220427005	All Individuals
Type A	Calls analyzed	Not recorded	2	1	Not recorded	3
	Call duration (ms)	/	25.30–64.70	52.7	/	25.30–64.70
	Inter-call intervals (ms)	/	NA	NA	/	NA
	Call rate	/	NA	NA	/	NA
	Note per call	/	1	1	/	1
	Pulse number	/	2.00–4.00	4	/	2.00–4.00
	Pulse rate	/	39.53–46.37	56.93	/	39.53–56.93
	Dominant frequency (kHz)	/	4.13–4.48	4.82	/	4.13–4.82
Type B	Calls analyzed	33	43	30	30	136
	Call duration (ms)	315.84 ± 35.11	289.86 ± 35.05	284.39 ± 16.94	274.06 ± 13.22	291.47 ± 31.59
		229.30–382.70	209.10–343.30	251.20–331.90	256.40–309.90	209.10–382.70
	Inter-call intervals (ms)	411.15 ± 103.38	420.35 ± 193.01	388.39 ± 99.26	470.82 ± 174.18	422.64 ± 154.88
		297.74–716.47	182.85–975.99	245.01–768.02	269.19–973.05	182.85–975.99
	Call rate	NA	86.33	90.93	82.28	82.28–90.93
	Note per call	1	1	1	1	1
	Pulse number	18.00[a] ± 1.50[b]	24.00 ± 3.50	25.00 ± 0.50	25.00 ± 1.13	24.00 ± 2.50
		13.00–21.00	15.00–34.00	20.00–29.00	21.00–28.00	(13.00–34.00)
	Pulse rate	53.89 ± 4.53	79.03 ± 10.74	83.39 ± 6.09	87.43 ± 6.02	75.75 ± 14.83
		46.40–64.05	59.80–105.19	70.29–99.04	75.34–97.93	46.40–105.19
	Dominant frequency (kHz)	4.38 ± 0.19	4.45 ± 0.09	4.55 ± 0.14	4.62 ± 0.07	4.49 ± 0.15
		4.13–4.65	4.13–4.48	4.48–4.82	4.48–4.65	4.13–4.82
Type C	Calls analyzed	31	63	3	41	138
	Call duration (ms)	191.85 ± 38.02	155.22 ± 8.11	159.20–192.30	207.83 ± 41.95	179.49 ± 37.56
		152.70–277.30	138.30–172.10		154.50–284.60	138.30–284.60
	Inter-call intervals (ms)	176.10 ± 28.97	144.92 ± 8.19	NA	187.96 ± 15.02	164.43 ± 25.60
		136.97–260.22	128.20–164.87		162.39–230.23	128.20–260.22
	Call rate	174.87	201.46	NA	157.13	157.13–201.46
	Note per call	2	2	2	2	2
	First note duration (ms)	40.35 ± 17.21	30.39 ± 9.19	24.85–35.78	62.09 ± 19.47	42.00 ± 20.04
		24.54–73.37	23.36–62.11		31.51–100.96	23.36–100.96
	Second note duration (ms)	65.32 ± 12.67	55.05 ± 10.24	47.22–70.97	52.57 ± 5.03	56.76 ± 10.77
		47.43–92.41	25.96–81.40		40.94–61.03	25.96–92.41
	Inter-note intervals (ms)	86.19 ± 31.72	69.78 ± 13.43	63.60–101.50	93.18 ± 29.46	80.73 ± 25.87
		45.20–159.90	36.20–99.20		52.70–157.00	36.20–159.90
	Note rate	10.77	12.92	11.57	9.99	9.99–12.92
	First note pulse number	2.00 ± 0.50	2..00 ± 0.00	2.00–3.00	4.00 ± 0.50	2.00 ± 1.00
		2.00–4.00	2.00–3.00		3.00–6.00	2.00–6.00
	Second note pulse number	4.00 ± 1.00	4.00 ± 0.50	3.00–5.00	4.00 ± 0.50	4.00 ± 0.50
		3.00–5.00	2.00–5.00		3.00–5.00	2.00–5.00
	Pulse rate	29.15 ± 6.40	31.53 ± 4.32	25.13–41.10	36.91 ± 6.46	32.58 ± 6.29
		18.83–39.22	19.34–43.86		26.61–48.08	18.83–48.08
	Dominant frequency (kHz)	4.27 ± 0.07	4.48	4.48–4.82	4.58 ± 0.09	4.47 ± 0.13
		4.13–4.31			4.48–4.65	4.13–4.82
Type D	Calls analyzed	Not recorded	12	28	5	45
	Call duration (ms)	/	276.86 ± 63.23	320.00 ± 40.88	242.60–282.90	302.22 ± 50.97
			220.50–442.00	253.20–402.70		220.50–442.00
	Inter-call intervals (ms)	/	NA	NA	NA	NA
	Call rate	/	NA	NA	NA	NA
	Note per call	/	2	2	2	2
	First note duration (ms)	/	27.92 ± 7.66	51.38 ± 20.10	19.03–39.48	42.75 ± 19.93
			13.22–45.19	18.24–80.09		13.22–80.09
	Second note duration (ms)	/	194.04 ± 40.14	225.17 ± 42.37	182.55–214.70	213.97 ± 41.71
			148.19–262.35	156.69–299.04		148.19–299.04
	Inter-note intervals (ms)	/	54.88 ± 27.51	43.45 ± 12.49	29.00–40.30	45.49 ± 18.10
			26.80–137.10	24.70–72.20		24.70–137.10
	Note rate	/	7.50	6.35	7.61	6.35–7.61
	First note pulse number	/	2.00 ± 0.00	4.00 ± 1.00	2.00–3.00	3.00 ± 1.50
			1.00–3.00	2.00–6.00		1.00–6.00
	Second note pulse number	/	10.50 ± 2.50	17.00 ± 2.50	14.00–17.00	16.00 ± 3.00
			9.00–21.00	9.00–21.00		9.00–21.00
	Pulse rate	/	49.40 ± 11.45	60.09 ± 5.95	61.83–73.27	57.89 ± 9.31
			27.15–71.75	38.36–67.57		27.15–73.27
	Dominant frequency (kHz)	/	4.48	4.76 ± 0.13	4.65	4.67 ± 0.16
				4.48–4.82		4.48–4.82

[a] Median instead of mean.
[b] Interquartile range instead of SD.

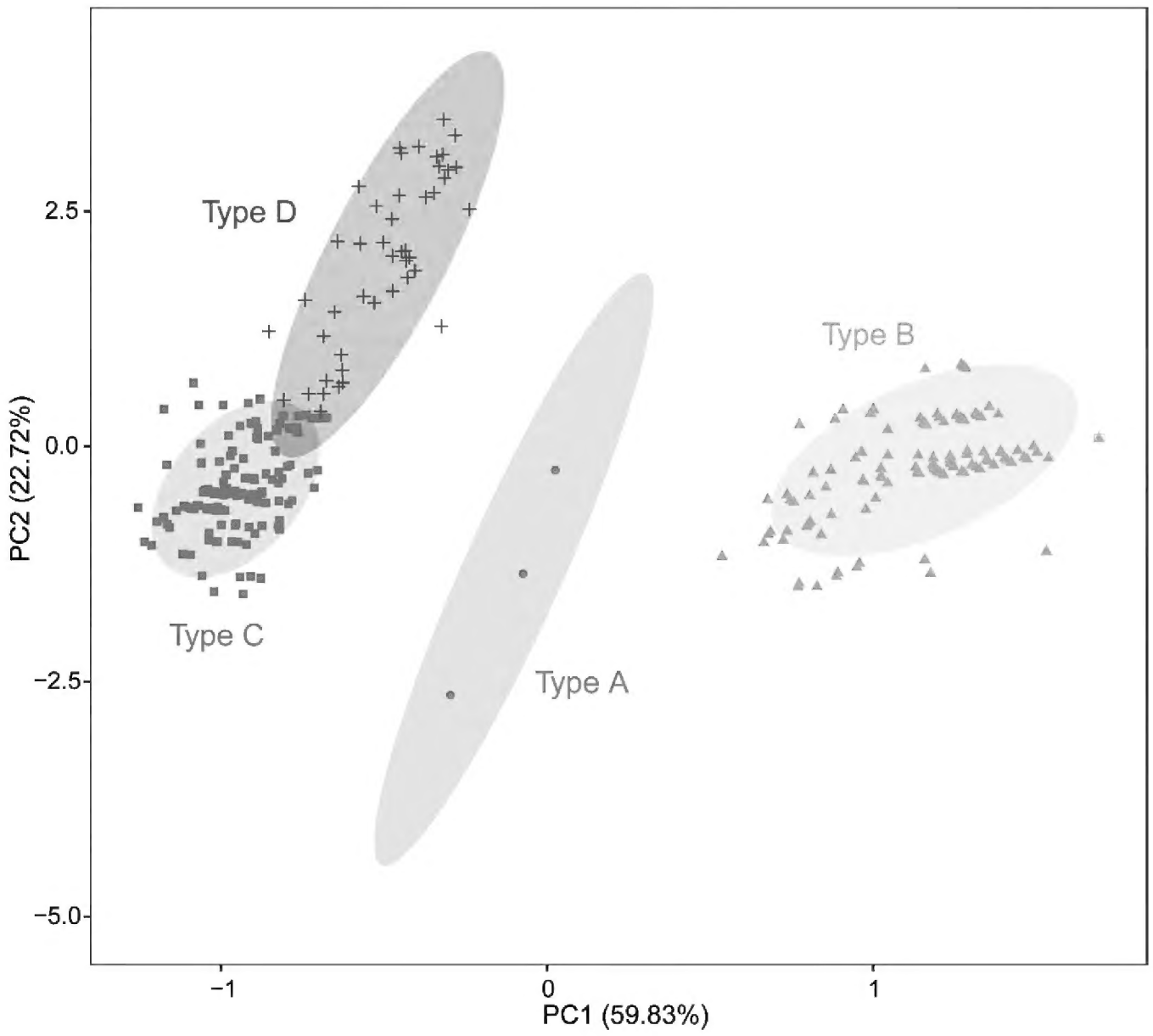

Figure 3. Plots of the first principal component (PC1) versus the second (PC2) for four types of advertisement calls of *Leptobrachella suiyangensis* from a principal component analysis.

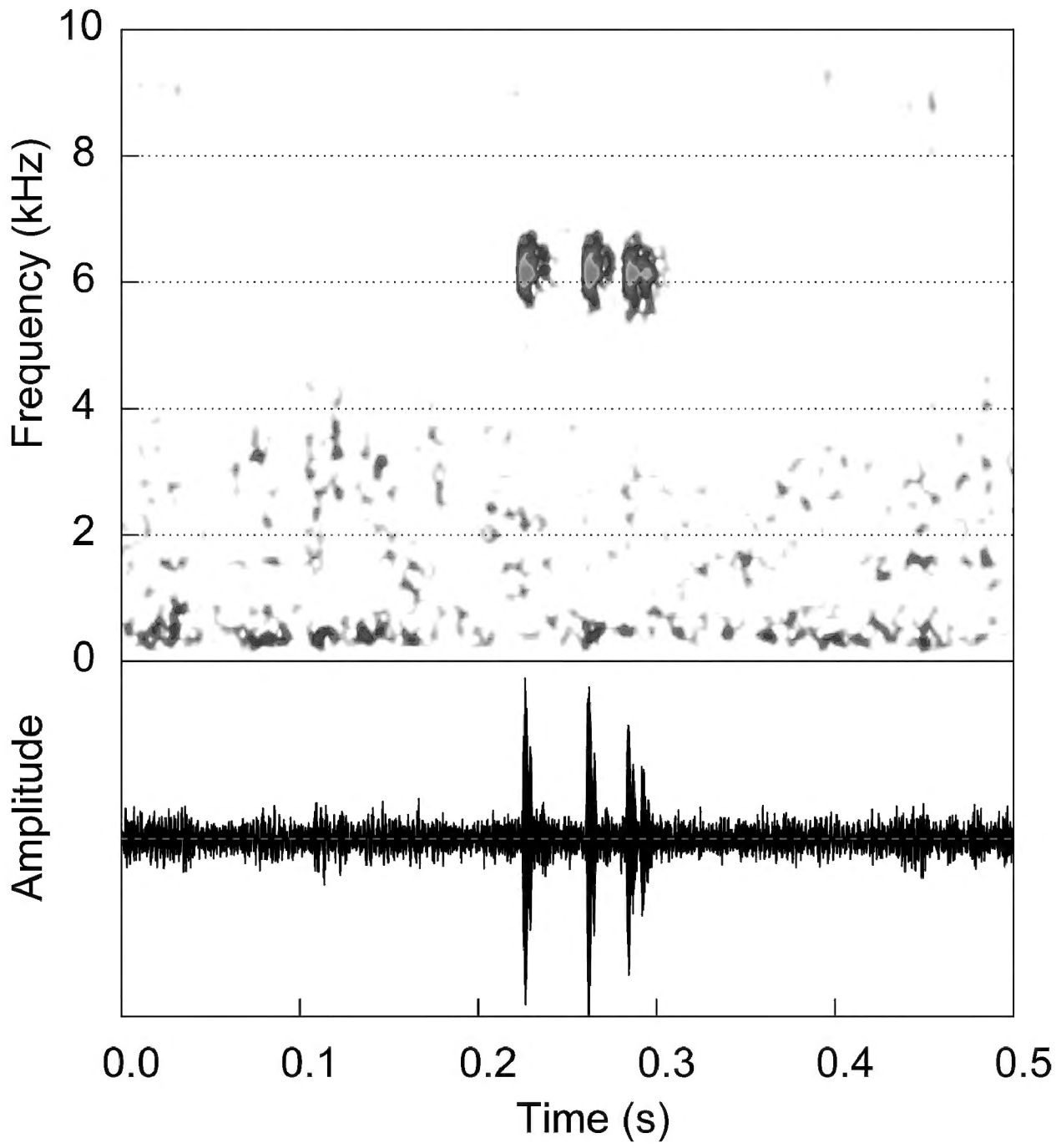

Figure 4. Oscillogram, spectrogram of the advertisement call of the *Leptobrachella bashaensis* (CJ20220501001, SVL 25.2 mm, 16.3 °C air temperature, 83% ambient humidity).

Table 4. Factor loadings of the Principal Component Analysis (PCA) on the advertisement call parameters of the *Leptobrachella suiyangensis*.

Call parameters	Principal components	
	1	2
Call duration	0.821	0.092
Dominant frequency	0.258	0.946
Note per call	-0.836	0.386
Note pulse number	0.987	0.02
Pulse rate	0.939	-0.019

Therefore, the significant differences in the call structure and type between *L. bashaensis* and *L. oshanensis* species groups may be related to their genetic distance.

Due to the diverse types of advertisement calls in *Leptobrachella*, Rowley et al. (2013) defined the primary advertisement call (PAC) and secondary advertisement call (SAC). These terms have been used in subsequent research on advertisement calls of *L. petrops*, *L. puhoatensis* and *L. tengchongensis*, etc (Rowley et al. 2017a, b; Yeung et al. 2021). Rowley et al. (2013) consider that the more frequent and higher amplitude clicking calls are referred to here as "primary calls", and low amplitude "rasps" are referred to as "secondary calls". Secondary calls were of less amplitude than primary calls, and not strongly amplitude modulated. But there is a similar dominant frequency between the "primary calls" and "secondary calls". However, the terms PAC and SAC do not apply to the advertisement calls of *L. suiyangensis*, as the advertisement calls of *L. suiyangensis* are more complex. Hence, we refer to Shi et al. (2021) to divide the advertisement calls of *L. suiyangensis* into four types. The difference in the structure of advertisement calls may be related to the phylogeny of the *Leptobrachella*, the *L. oshanensis* species group belongs to lineage A1 of the genus, while *L. petrops* belongs to lineage A3 and *L. botsfordi* belongs to lineage C (Chen et al. 2018). The differentiation of advertisement calls in *Leptobrachella* can serve as evidence for their differentiation in phylogenetic relationships.

Table 5. Descriptive statistics for acoustic characteristics of advertisement calls of *Leptobrachella bashaensis*. NA = not applicable.

Vouchers	CJ20220501001	CJ20220501003	CJ20220501005	All Individuals
Calls analyzed	40	30	30	100
Call duration (ms)	66.68 ± 6.60	65.78 ± 6.24	65.34 ± 7.87	66.01 ± 6.86
	51.20–79.80	51.30–78.80	48.00–78.50	48.00–79.80
Inter-call intervals (ms)	318.73 ± 52.10	323.61 ± 54.01	366.89 ± 81.25	334.59 ± 65.61
	257.69–468.88	264.25–468.42	259.61–538.39	257.69–538.39
Call rate	158.96	158.48	142.86	142.86–158.96
Note per call	1	1	1	1
Note rate	NA	NA	NA	NA
Note pulse number	3.00 ± 0.38	3.00 ± 0.00	3.40 ± 0.50	3.00 ± 0.50
	3.00–4.00	3.00–4.00	3.00–4.00	3.00–4.00
Pulse rate	33.55 ± 3.82	33.37 ± 4.36	36.55 ± 4.72	34.40 ± 4.46
	29.28–40.65	28.78–46.08	30.08–47.69	28.78–47.69
Dominant frequency (kHz)	6.18 ± 0.07	6.20 ± 0.06	6.10 ± 0.08	6.16 ± 0.08
	6.03–6.29	6.12–6.29	6.03–6.46	6.03–6.46

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Supplementary material 1

Summary of male advertisement call parameters of *Leptobrachella suiyangensis*, *Leptobrachella bashaensis* and 16 other species

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Data type: xls

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